REFERENCES

Clutterbuck, P. W. & Raistrick, H. (1933). *Biochem. J.* **27**, 654.
Cohen, S., Snyder, J. C. & Mueller, J. H. (1941). *J. Bact.* **41**, 581.
Dagley, S. & Hinshelwood, C. N. (1938). *J. chem. Soc.* p. 1930.
Davies, D. S. & Hinshelwood, C. N. (1943). *Trans. Faraday Soc.* **39**, 431.
Davies, D. S., Pryce, J. M. & Hinshelwood, C. N. (1944). *Trans. Faraday Soc.* **40**, 34.
Florey, H. W., Gilliver, K., Jennings, M. A. & Sanders, A. G. (1945). In the Press.
Hadley, P. (1937). *J. infect. Dis.* **60**, 129.
Heatley, N. G. (1944). *Biochem. J.* **38**, 61.
Hinshelwood, C. N. (1944). *Biol. Rev.* **19**, 135.
Hinshelwood, C. N. & Lodge, R. M. (1944). *Proc. roy. Soc.* B, **132**, 47.
Hoffmann, C. E. & Rahn, O. (1944). *J. Bact.* **47**, 177.
Knox, R. & Pollock, M. R. (1944). *Biochem. J.* **38**, 299.
Lodge, R. M. & Hinshelwood, C. N. (1943). *J. chem. Soc.* p. 213.
Lodge, R. M. & Hinshelwood, C. N. (1944). *Trans. Faraday Soc.* **40**, 571.
Mueller, J. H. (1939). *Proc. Soc. exp. Biol., N.Y.*, **40**, 632.
Stephenson, M. & Yudkin, J. (1936). *Biochem. J.* **30**, 506.
Wilkins, W. H. & Harris, G. C. M. (1943). *Brit. J. exp. Path.* **24**, 141.
Wilson, G. S. (1922). *J. Bact.* **7**, 405.
Wyckoff, R. W. G. (1934). *J. exp. Med.* **59**, 381.
Yudkin, J. (1938). *Biol. Rev.* **13**, 93.

Metabolism of Acetoacetate in Animal Tissues. 1

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The utilization of acetoacetic acid in animal tissues is commonly ascribed to two types of metabolic processes: reduction to β-hydroxybutyric acid and oxidation to CO_2 and water. The reduction and its reversibility was first observed by Friedmann & Maase (1910) and Wakeman & Dakin (1910). The occurrence of an oxidative breakdown became established when Magnus-Levy and Knoop showed that acetoacetic acid is a normal intermediate in the decomposition of fatty acids. It was further demonstrated in conclusive experiments by Snapper & Grünbaum (1927). This pioneer work was later confirmed and amplified by many workers, especially when the tissue slice technique was applied (Jowett & Quastel, 1935; Quastel & Wheatley, 1935; Edson & Leloir, 1936). Reference to details of this work will be made later in this paper in connexion with our own findings.

As yet, relatively little is known about the intermediary steps in which acetoacetate disappears. Krebs & Johnson (1937) showed that the reduction can be brought about by dismutation with α-ketonic acids:

$$\text{acetoacetate} + \alpha\text{-ketoglutarate} + H_2O \rightarrow \beta\text{-hydroxybutyrate} + \text{succinate} + CO_2,$$

$$\text{acetoacetate} + \text{pyruvate} + H_2O \rightarrow \beta\text{-hydroxybutyrate} + \text{acetate} + CO_2.$$

As for the mechanism of oxidation, Weil-Malherbe (1938) excluded α-hydroxyacetoacetate as an intermediate. Hoff-Jorgensen (1940) reported that erythro-1:2-dihydroxybutyrate is rapidly destroyed after intravenous injection, whilst little or none of the threo-form is attacked under the same conditions. Weil-Malherbe (1938), in contrast, found that the threo-form increases the O_2 uptake of, and is removed by, kidney cortex, whilst the erythro-form was inactive. Kleinzeller (1943) found that crotonate, vinylacetate, γ-hydroxybutyrate and *dl*-2:3-dihydroxybutyrate are oxidizable in kidney cortex. The substances which are metabolized are possibly, but not necessarily, normal intermediates; further experiments will be required to decide whether they are normal metabolites.

Breusch (1943) and apparently independently Wieland & Rosenthal (1943) recently suggested that the $-CH_2.CO_2H$ grouping of acetoacetate is transferred as a whole to oxaloacetate, resulting in the formation of citric acid:

$$CH_3.CO.CH_2.CO_2H + CO_2H.CO.CH_2.CO_2H + H_2O \rightarrow \text{citric acid} + \text{acetic acid (Breusch, 1943)}, \quad (1)$$

$$CH_3.CO.CH_2.CO_2H + 2CO_2H.CO.CH_2.CO_2H + H_2O \rightarrow 2 \text{ citric acid (Wieland \& Rosenthal, 1943)}. \quad (2)$$

These schemes attempt to explain the observation that under some conditions oxaloacetate and acetoacetate together form more citric acid than oxaloacetate alone.

In the present investigation an effort is made to examine as fully as possible the chemical changes which accompany the disappearance of acetoacetate from animal tissues. It is already known from previous work (Jowett & Quastel, 1935; Breusch, 1943) and was soon confirmed by our own experiments

that the removal of acetoacetic acid is in some tissues linked up with the metabolism of the C_4 dicarboxylic acids, especially fumarate, malate and oxaloacetate. Preliminary work indicated that the chief chemical changes associated with the removal of acetoacetate concern, apart from β-hydroxybutyrate, the di- and tricarboxylic acids of the tricarboxylic acid cycle. We determined therefore the changes in the concentrations of the following 15 substances: acetoacetate, β-hydroxybutyrate, the eight acids of the tricarboxylic acid cycle, pyruvate, lactate, α-hydroxyglutarate, O_2 and CO_2. In some experiments most of the above metabolites were estimated, in others only a few.

EXPERIMENTAL

Tissue material

Minced versus sliced tissues. Preliminary experiments on sheep kidney cortex showed that the rates of removal of acetoacetate in sliced and in minced tissue are under certain conditions of the same order of magnitude. We preferred to use minced tissues for most experiments because relatively large quantities of tissues—between 10 and 20 g. fresh weight—were frequently needed for the many different analyses. To slice these quantities would have required too much time. It is possible to mince 20 g. of tissue within a few minutes, whereas slicing the necessary amount would have taken at least 30–40 min. and might have resulted in loss of enzyme activity.

Choice of tissue. Mammalian heart muscle was found to have the highest rate of acetoacetate removal, $Q_{ac.-ac.}$ reaching values up to −30. Kidney cortex was about half as active as heart; in other tissues the rate of acetoacetate metabolism was very much lower. In pigeon breast muscle, for example, the rate is only of the order of magnitude of the non-enzymic decomposition and its accurate measurement is therefore difficult.

Most experiments were carried out on sheep heart muscle obtained from the abattoir. The animal was electrically stunned and bled through the carotids. Immediately afterwards the sternum was sawn apart and the heart, still beating, removed and placed in a jar containing ice and iced water. It was then transported to the laboratory which it reached within 20 to 40 min. The rapid removal of the heart from the animal and efficient cooling were essential for the preservation of the metabolic activities. The enzymes in heart muscle, like those of skeletal muscle, appear to be somewhat more labile than those in most other tissues—presumably because post-mortal changes, especially the lactic acid formation, are more rapid in cardiac and skeletal muscle than in other tissues.

Tissue suspensions. The tissue was minced in a Latapie mill, weighed and suspended evenly in 6·5 vol. of ice-cooled saline. The saline medium was calcium-free phosphate saline (Krebs & Eggleston, 1940). Three volumes of this suspension were measured into the experimental vessel and 1 vol. of further solutions was added—substrates, usually in 0·2 M solution and/or enough saline medium to bring the concentration of the tissue in the final suspension to 10% (w/v, calc. for fresh weight). For the calculation of Q values the dry weight was assumed to be one-fifth of the wet weight. When anaerobic conditions were required, the vessel was filled with N_2 and a stick of yellow phosphorus was placed in a special compartment of the vessel to remove traces of O_2. The suspension was shaken at 40° for specified periods. At the end of this period, ¼ vol. 2 N-HCl was added to stop metabolic processes. The suspension was then cooled in an ice-bath and stored in a refrigerator until it was required.

Vessels

Some experiments were carried out in conical Warburg flasks of about 20 ml. size, provided with a side-arm and a centre well. These were used when a small number of metabolites was to be analyzed, or when the O_2 uptake and the CO_2 output were to be measured. When larger amounts of tissue suspension were required for analytical purposes, either large conical manometer vessels of the dimensions shown in Fig. 1 were used, or flasks shown in Fig. 2, which resemble those described by Krebs (1932), with the addition of a centre well (to hold a stick of yellow phosphorus in anaerobic experiments) and the provision of a side-arm (from which substrates can be added after the complete elimination of O_2 by the phosphorus). These flasks were shaken in a water-bath at 40° as described by Krebs (1932).

Substrates

Oxaloacetic acid was prepared according to Wohl & Oesterlin (1901) and Wohl & Claussner (1907). It was dissolved and neutralized with M-$NaHCO_3$ immediately before use.

Preparation of acetoacetate. 2·6 ml. of recently distilled ethylacetoacetate and 10·2 ml. 2 N-NaOH were made up to 20 ml. with water and kept for 1 hr. at 40°. The solution was then neutralized with N-HCl (using litmus paper), washed into a distilling flask with 20 ml. water and reduced *in vacuo* to about 5 ml. in order to remove ethanol. The solution was transferred to a measuring flask and made up to 20 ml. This stock solution (about M) was stored in the refrigerator. Before use it was usually diluted to about 0·2 M. The exact strength of the solution used was remeasured in each experiment. The complete removal of ethanol was essential as it interferes with the determination of β-hydroxybutyric acid.

Spontaneous decomposition of acetoacetate. The occurrence of a non-enzymic 'ketone decomposition' of acetoacetate makes it difficult to estimate accurately the rate of acetoacetate metabolism when the rate of the latter is low. The spontaneous decomposition depends, among other factors, on the acetoacetate concentration and on the presence of amino compounds (Pollak, 1907; Ljunggren, 1925) and varies therefore with the conditions of the experiment.

We tried to measure the spontaneous decomposition by separating it from the metabolic decomposition by heat inactivation. The tissue suspension was warmed for 60 min. to 60°; this treatment suppresses the metabolic processes and any disappearance of acetoacetate from such an 'inactivated' suspension may therefore be attributed to non-enzymic reactions. According to Table 1 the loss of acetoacetate in sheep heart suspension of the type used in our experiments was 4–5% after 40 min. and 6·4% after 80 min. incubation. The losses recorded in the table included that incurred during the equilibration period of the manometric estimation—between 1 and 2%—and this circumstance may explain why the loss is not directly proportional

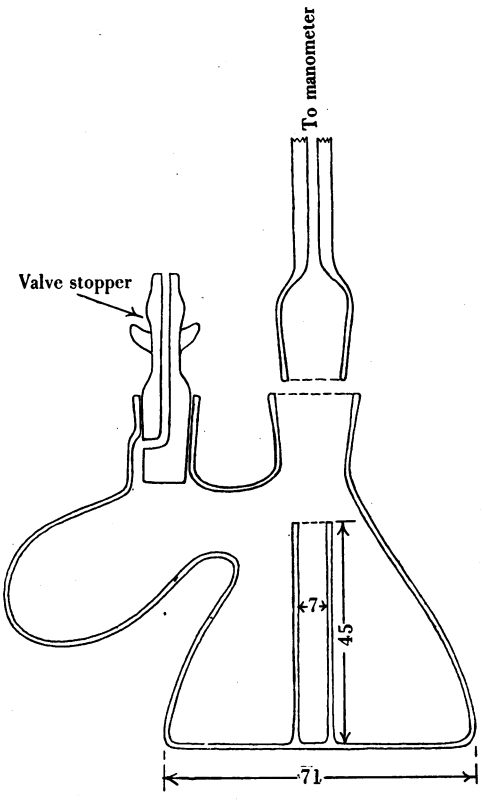

Fig. 1.

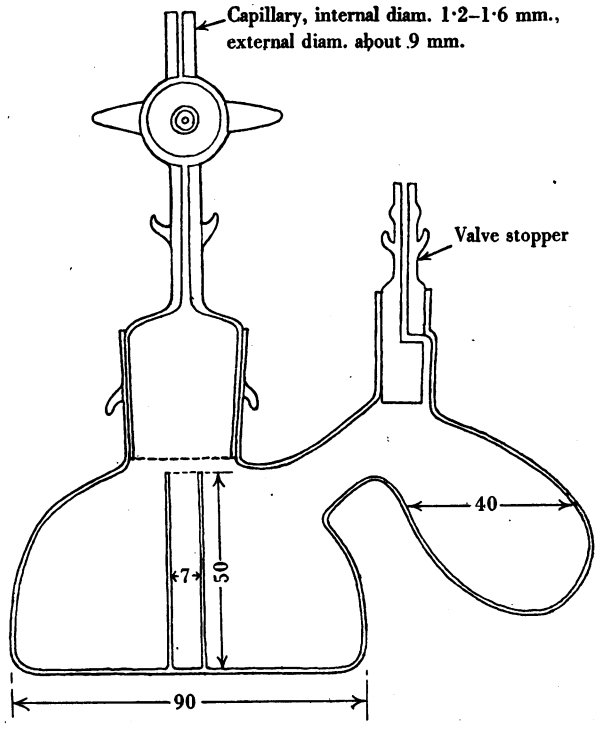

Fig. 2.

Fig. 1. Manometric flasks for large-scale experiments, holding up to 25 ml. fluid in aerobic and 60 ml. in anaerobic experiments. The side-arm holds at least 10 ml. fluid without danger of spilling when shaken in the water-bath. Total volume 110–120 ml. Dimensions in mm. ¼ actual size.

Fig. 2. Flask for metabolic experiments, with side-arm, centre tube (for NaOH or yellow phosphorus) and gas in- and outlets. The side-arm holds at least 12 ml. fluid without danger of spilling when shaken. Total volume about 250 ml. Dimensions in mm. ¼ actual size.

Table 1. *Recovery of acetoacetate added to metabolically inactive tissue*

(Tissue minced, suspended in 9 parts phosphate saline and inactivated by heat; duplicate determinations; the data refer to 4 ml. suspension; 40°.)

		Acetoacetate			
Tissue	Period of incubation (min.)	Added (μl.)	Recovered (μl.)	Loss (μl.)	Mean loss (% of that added)
Sheep heart	40	1760	1665	− 95	5·1
	40	1760	1675	− 85	
	40	2265	2185	− 80	4·0
	40	2265	2165	−100	
	80	2265	2110	−155	6·4
	80	2265	2130	−135	
Sheep kidney	40	1760	1625	−135	7·4
	40	1760	1635	−125	

to the duration of the incubation. In kidney suspensions the loss was somewhat greater than in heart suspensions.

Throughout this paper the data on the disappearance of acetoacetate represent the actual measurements, no correction for the non-enzymic loss having been made, as this loss is usually small; but attention is drawn to the loss where it is of significance in the interpretation of the results.

Analytical methods

Acetoacetic and oxaloacetic acids. The separate manometric determination of these two β-ketonic acids, when present together, is based on the fact that aniline decarboxylates both acids, whilst Al^{+++} salts decarboxylate oxaloacetic acid only (Krebs, 1942). Both determinations were carried out directly on the supernatant of the centrifuged tissue suspension.

The aniline method for the determination of β-ketonic acids (Ostern, 1933; Edson, 1935) was modified in order to reduce the 'spontaneous' decomposition of the β-ketonic acids during the period of equilibration. The rate of the spontaneous decomposition is lowered by mineral acids,

the pH optimum being near the neutral point (Ljunggren, 1925). We therefore add enough 2N-HCl to the solution to bring its final concentration to about 0·2N. The main compartment of the vessels was filled with 3 ml. of the unknown solution (containing up to 400μl. β-ketonic acid), 0·3 ml. 2N-HCl and 0·5 ml. 50% citric acid, the side-arm with 1 ml. aniline citrate (4·5 ml. aniline plus 5·5 ml. 50% citric acid). The temperature of the bath was 25°. On mixing the reagents in the absence of β-ketonic acids a negative pressure of 14 mm. Brodie fluid was observed on the manometers. Allowance for this 'blank' was made in all measurements.

Oxaloacetic acid was determined in conical Warburg flasks provided with two side-arms, each capable of holding at least 1 ml. fluid. The main compartment contained 2 ml. of the solution to be tested, acidified with 2N-HCl to 0·2N. Side-arm one contained 1 ml. 0·75M-phthalate buffer (15·3 g. potassium hydrogen phthalate and 1·8 g. NaOH in 100 ml.), side-arm two 1 ml. 33·3% $Al_2(SO_4)_3.18H_2O$. The bath temperature was 25°. After equilibration the contents, first of side-arm one, and then of side-arm two, were mixed with the solution in the main compartment. A control experiment, measuring the changes of pressure due to mixing the various solutions and containing water instead of the unknown solution, was run parallel with the estimations. The CO_2 evolution was usually complete within 60 min. The yields were a few per cent below the theoretical amounts, probably owing to the spontaneous decomposition of oxaloacetic acid during the period of equilibration. For example, 215 and 107·5μl. were recovered from pure aqueous solutions containing 224 and 112μl. acid. In another recovery experiment, oxaloacetic acid was added to muscle extract containing 0·2N-HCl and the period of equilibration was extended to 1 hr. (usually 20 min. are required); the yields were in this case between 87·5 and 89%. The amounts of acetoacetic acid were calculated by deducting the amounts of oxaloacetic acid from those of total β-ketonic acids as determined by the aniline method.

Lactic acid was determined according to Friedemann & Graeser (1933). A suitable quantity of tissue suspension containing preferably about 2 mg. lactic acid was diluted to 40 ml. and protein and other interfering substances were removed by the copper-lime precipitation. Controls showed that malic, fumaric, citric, pyruvic, oxaloacetic and β-hydroxybutyric acids, in the quantities occurring in our samples, did not significantly interfere. Acetoacetic acid and acetone were removed by boiling the solution gently in the distilling flasks for 10 min. before $KMnO_4$ was added and before the distillate was collected.

Pyruvic acid was determined by the carboxylase method. 1 ml. of the acid (0·4N) tissue suspension, 0·4 ml. 3M-sodium acetate—to adjust the pH to 5·0—and 2 ml. water were measured into the main compartment of a conical manometer flask, and 1 ml. freshly prepared yeast extract (see Krebs & Johnson, 1937) was placed in the side-arm. If oxaloacetic acid was present the result was corrected by assuming that 1 molecule of oxaloacetic acid, when added to carboxylase, yielded 1·0 mol. CO_2 (this being the average of eight determinations). In most determinations a rapid CO_2 evolution from the decarboxylation of pyruvic and oxaloacetic acids was followed by a slow and steady evolution of CO_2, probably from α-ketoglutaric acid. The end-point of the rapid decarboxylation was ascertained by extrapolation.

Succinic acid was extracted with ether after deproteinization with tungstic acid and measured manometrically (Krebs, 1937). Usually 3–5 ml. suspension were used. The ether for the extraction must be free from aldehyde (2 ml. Nessler's reagent must remain clear when shaken with 5 ml. ether), the presence of which causes loss of succinic acid. Small amounts of acetaldehyde were removed by shaking the ether with alkaline $KMnO_4$ (saturated solution of $KMnO_4$ in N-NaOH).

α-Ketoglutaric and α-hydroxyglutaric acids. As cardiac muscle reduces α-ketoglutaric acid to α-hydroxyglutaric acid (Weil-Malherbe, 1937), the two acids are likely to occur together. The sum of the acids was measured as follows (see Krebs, 1938). A portion of the protein-free filtrate (deproteinized with tungstic acid) corresponding to about 4 or 5 ml. tissue suspension, was diluted to about 15 ml. and acidified with 2 ml. 50% H_2SO_4. Then first 5 ml. 4·7% $KMnO_4$ and afterwards 0·5 ml. acid $MnSO_4$ were added and allowed to react for 60 min. at room temperature. The excess of oxidizing agent was removed by addition of enough acid $FeSO_4$ solution (200 g. $FeSO_4.7H_2O$ and 10 g. H_2SO_4 in 1 l.) to turn the colour of the solution pale green. The succinic acid formed was extracted and determined. When succinate was present in the original suspension, this was determined in a separate portion. The difference between succinic acid found after direct extraction and succinic acid found after treatment with $KMnO_4$ represents the sum of α-keto- and α-hydroxyglutarate. Glutamic acid does not react in significant quantities under the above conditions. Using this procedure we recovered about 95% α-ketoglutarate and about 90% α-hydroxyglutarate from pure solutions.

For the separate determination of α-ketoglutaric and α-hydroxyglutaric acids an excess of 2:4-dinitrophenylhydrazine (1% solution in 10% H_2SO_4) was added to the deproteinized filtrate. After standing 30 min., the hydrazones were extracted with aldehyde-free ether and oxidized to succinic acid as previously described (Krebs, 1938). Before the extraction of succinic acid, $FeSO_4$ solution was added as stated above. The aqueous phase containing α-hydroxyglutaric and succinic acids was mixed with 1 ml. acetone to remove the excess dinitrophenylhydrazine. The volume was measured and the precipitate filtered off after 30 min. A known quantity of the filtrate was boiled to remove the excess of acetone. After cooling, the solution was acidified, treated with $KMnO_4$ and extracted as stated for the determination of the sum of the keto- and hydroxy-acids. Recovery from pure mixtures of the two acids was about 95% for α-ketoglutaric acid and 75% for α-hydroxyglutaric acid.

Fumaric and malic acids. Fumaric acid was estimated by the method of Krebs, Smyth & Evans (1940), by reduction to succinic acid and manometric determination of the latter using 3–5 ml. suspension. In previous work from this laboratory the concentration of *l*(−)-malic acid was calculated from the equilibration constant on the assumption that owing to the powerful activity of fumarase the two acids are virtually present as the equilibrium mixture. This assumption was based on the observation that under experimental conditions the equilibrium is established within a few minutes, 5–10 min. when fumarate was added (see Table 2), and even more rapidly when *l*(−)-malate was added. In these experiments, however, *sec.*-octanol had been added to the suspensions in order to eliminate oxi-

Table 2. *Rate of conversion of fumarate into l(−)-malate in sheep heart*

(6 g. (fresh weight) minced sheep heart (frozen and stored for 6 days), 48 ml. phosphate saline; 6 ml. 0·1 M-fumarate, one drop octanol, incubated at 40°. *l*(−)-Malate estimated polarimetrically at intervals in 8 ml. samples.)

Period of incubation (min.)	$[\alpha]_D$ observed (°)	*l*(−)-Malic acid formed (mg./ml. solution)
2·5	0·95	0·77
5	1·12	0·90
10	1·18	0·95
20	1·19	0·96*

* On the assumption that the ratio *l*(−)-malate/fumarate, in the equilibrium mixture is 3·17 (Krebs, Smyth & Evans, 1940), 1·02 mg. *l*(−)-malic acid are expected.

dative enzymes. In the course of the present work we found that the proportion of fumaric to malic acids in the metabolically active tissue differed frequently from the expected ratio. The ratio malate/fumarate was usually much lower than it is in tissue from which the energy-giving reactions have been eliminated. For example, when a suspension of fresh sheep heart in 9 parts of phosphate saline was shaken for 30 min. with fumarate (final concentration 0·02 M) the following ratios were observed (40°):

Shaken in O_2: ratio malate/fumarate, 1·75; 1·56 (duplicates).

Shaken in N_2: ratio malate/fumarate, 1·53; 1·65 (duplicates).

When the same cardiac muscle was used after washing with water and addition of octanol, the ratio measured under otherwise identical conditions agreed within the limits of error with the previously reported figure of 3·17. It was thus not permissible to calculate the concentration of malic acid from the equilibrium constant. We used the polarimetric method of Auerbach & Krüger (1923), as modified by Krebs & Eggleston (1943), for the independent determination of malic acid; 8 ml. unknown solution which contained 0·2 N-HCl, 2 ml. M-trisodium citrate, 1 ml. glacial acetic acid, 29% (w/v) aqueous commercial ammonium molybdate, freshly prepared solution, and about 0·5 g. charcoal, were mixed and filtered and the rotation was read in 2 dm. tubes. Under the conditions employed $[\alpha]_D^{20°}$ was +1550° and a reading of 1° indicated the presence of 0·806 mg. malic acid/ml. of the unknown solution under the same conditions. $[\alpha]_D^{20°}$ of the molybdate complexes of *l*(+)-lactic acid was −84°; of *l*(−)-α-hydroxyglutaric acid −76°; of (−)-*iso*citric acid −780°. The quantities of these three acids in our solutions were usually so small that their effect on the rotation could be neglected. In some cases correction factors based on the above figures were used.

As for the explanation of the differences of the ratios malate/fumarate in fresh and treated tissue, the assumption that the differences are due to the circumstance that the two reactions establishing the equilibrium are outpaced by other reactions of fumarate and malate upsetting the equilibrium is not supported by the available evidence; fumarase seems to be fully active in fresh tissue; the potential rates of the reactions catalyzed by this enzyme, therefore, are at least 10 times greater than the rates of other reactions of fumarate and malate. It is more probable that the environment of the enzyme is different in fresh and treated tissue. The enzyme may be localized in a lipid phase where the relative concentrations of the two acids and of water are different. Jacobsohn (1934), Jacobsohn, Pereira & Tapadinhas (1932) and Jacobsohn & Tapadinhas (1934) also noted that the equilibrium depends on the source and treatment of the enzyme preparation.

β-Hydroxybutyric acid. A modification of Van Slyke's mercuric sulphate method was used (see Edson, 1935). A quantity of the solution containing preferably between 1 and 2 mg. of the acid was diluted to 40 ml. in a stoppered 50 ml. measuring cylinder and mixed with 5 ml. 20% (w/v) $CuSO_4.5H_2O$ and 5 ml. 20% (w/v) $Ca(OH)_2$ suspension. After 30 min. the suspension was filtered and 30 ml. filtrate were transferred to a 250 ml. ground-glass boiling flask; 2·4 ml. 50% (v/v) H_2SO_4 and 8·4 ml. 10% (w/v) $HgSO_4$ were then added. The solutions were boiled under reflux for 30 min. and then allowed to stand overnight. The precipitate, containing the mercuric complexes of acetone, pyruvic acid, and possibly of other substances, were separated from the solution by suction through a Jena G4 sintered glass funnel and washed with a few ml. 5% sulphuric acid. The further treatment was as described by Edson (1935) except that an extra amount—0·5 ml.—of 7·5 N-NaOH was added to facilitate the reduction of the Hg ions, and 10 min. were allowed for the reduction. After addition of iodine, the solution was allowed to stand until it was completely clear (30–60 min.). Recovery of β-hydroxybutyrate added to inactivated tissue suspensions was quantitative.

This method, like all other micro-methods for the determination of β-hydroxybutyric acid, suffers from lack of specificity. However, we have convinced ourselves that interfering substances, among them lactic acid and ethanol, did not occur in significant quantities in our material, for β-hydroxybutyric acid was found only when acetoacetic acid had been added. In control suspensions similar quantities of substances which might be expected to interfere—citric, malic, fumaric, α-ketoglutaric, lactic acids—yielded no measurable amount of 'β-hydroxybutyric acid'.

Citric, l(−)-*isocitric and* cis-*aconitic acids* were determined according to the methods described by Krebs & Eggleston (1944). The colorimetric procedure was followed whenever β-hydroxybutyric acid was present or likely to be present. The three tricarboxylic acids were always found to be present as their equilibrium mixture.

Total CO_2 was measured manometrically according to Warburg & Yabusoe (1924).

RESULTS

Aerobic and anaerobic removal of acetoacetate. Data showing the order of magnitude of the rate of acetoacetate removal in minced sheep heart are shown in Table 3. The data all refer to suspensions where acetoacetate was the only substrate added. It will be seen that under these conditions two to three times more acetoacetate is removed aerobically than anaerobically.

Effect of malonate. Jowett & Quastel (1935), working with kidney tissue, found that the aerobic removal of acetoacetate is inhibited by malonate

Table 3. *Removal of acetoacetate under aerobic and anaerobic conditions in sheep heart*

(The data refer to 4 ml. suspension.)

Exp. no.	Initial amount of aceto-acetate (μl.)	Period of incu-bation (min.)	Final amount of acetoacetate (μl.) Aerobic	Anae-robic	$Q^{O_2}_{ac.-ac.}$	$Q^{N_2}_{ac.-ac.}$
1	1776	30	1073	1535	−17·6	−6·0
2	1752	30	1233	—	−13·0	—
3	1752	30	1220	1490	−13·3	−6·6
4	908	40	—	679	—	−4·3
5	888	40	—	604	—	−5·3
6	1866	30	—	1546	—	−8·0
7	1814	30	—	1432	—	−9·6
8	1732	40	1145	1375	−11·4	−7·1

whilst the anaerobic removal is not affected. This is also true for minced sheep heart, as will be seen from Table 4: even 0·001 M-malonate has a powerful effect. Malonate reduces the rate of the aerobic to that of the anaerobic removal.

Table 4. *Effect of malonate on the removal of acetoacetate in sheep heart*

(0·02 M-acetoacetate; 40 min. incubation; the data refer to 4 ml. suspension.)

Final concentration of substances added	Gas	Acetoacetate removed from 4 ml. medium (μl.)	$Q_{ac.-ac.}$
—	O_2	703	−17·6
0·001 M-malonate	O_2	258	− 6·5
0·01 M-malonate	O_2	166	− 4·2
—	N_2	241	− 6·0
0·01 M-malonate	N_2	286	− 7·2

Inhibitions by low concentrations of malonate suggest—if not prove—that succinic dehydrogenase plays a part in the removal of acetoacetate. This assumption served as the working hypothesis for the further experiments.

Acceleration of acetoacetate removal. It seemed probable, bearing in mind the relations between succinic dehydrogenase and pyruvate metabolism in muscle tissue (Krebs & Eggleston, 1940), that the removal of acetoacetate depends on substances formed from succinate; the effect of the various acids of the tricarboxylic acid cycle was therefore examined. Since the substances are interconvertible under aerobic conditions, no clear-cut results can be expected in the presence of O_2 and anaerobic conditions were therefore chosen. These do not completely exclude interconversion of some of the members of the cycle, especially in the presence of an excess of oxaloacetate, but they reduce the possibilities of interconversion.

Four of the eight acids of the cycle—succinate, citrate, *iso*citrate, *cis*-aconitate—had no effect on the anaerobic removal of acetoacetate; the other four—fumarate, *l*(−)-malate, oxaloacetate and α-ketoglutarate—greatly increased the rate (Tables 5 and 6) and raised it to, or even above, the level of the aerobic removal.

The increase was also observed under aerobic conditions, but the effect was less marked. When malonate is present, however, the four acids increased the aerobic acetoacetate removal almost as much as the anaerobic removal. They almost abolished the malonate inhibition (Table 6). These results support the view that metabolites arising in the tissue through the intermediation of succinic

Table 5. *Acceleration of the anaerobic removal of acetoacetate in sheep heart*

(The data refer to 4 ml. suspension.)

Exp. no.	Gas	Initial concentration of aceto-acetate (M)	Substance added	Final concentration (M)	Period of incubation (min.)	Aceto-acetate removed (μl.)	$Q_{ac.-ac.}$
1	N_2	0·01	—	—	20	193	− 7·2
			Fumarate	0·01		494	−18·5
			l(−)-Malate	0·01		450	−16·9
			Oxaloacetate	0·01		456	−17·1
			α-Ketoglutarate	0·01		334	−12·5
2	N_2	0·02	—	—	30	241	− 6·0
			Fumarate	0·005		768	−19·2
3	N_2	0·02	—	—	30	262	− 6·6
			Oxaloacetate	0·02		667	−16·7
			α-Ketoglutarate	0·02		717	−17·9
4	N_2	0·02	—	—	40	377	− 7·1
			Oxaloacetate	0·01		1062	−19·9
			Fumarate	0·01		1339	−25·1
			α-Ketoglutarate	0·01		819	−15·3
5	O_2	0·02	—	—	40	607	−11·4
			Fumarate	0·01		1092	−20·5
			Oxaloacetate	0·01		1114	−20·9
			α-Ketoglutarate	0·01		877	−16·4

dehydrogenase play a part in the removal of acetoacetate.

Table 6. *Effect of fumarate, oxaloacetate and α-ketoglutarate on the removal of acetoacetate by sheep heart, in the absence and presence of malonate*

(0·02 M-acetoacetate; 60 min. incubation; the data refer to 4 ml. suspension.)

	Further substrates added			Acetoacetate removed (μl.)	
Exp. no.	Substance	Final concentration (M)	Gas	No malonate	With 0·01 M-malonate
1	None	—	O_2	950	120
	Fumarate	0·02	O_2	1212	924
	α-Ketoglutarate fumarate	0·02	O_2	1243	545
	α-Ketoglutarate	0·02	O_2	1472	1070
2	None	—	N_2	420	330
	α-Ketoglutarate	0·02	N_2	900	735
	Fumarate	0·02	N_2	1395	1192
	Oxaloacetate	0·02	N_2	1190	1185
	l(−)-Malate	0·02	N_2	1612	1240
3	None	—	O_2	870	148
	α-Ketoglutarate	0·02	O_2	838	363
	Fumarate	0·02	O_2	1068	858
	Oxaloacetate	0·02	O_2	805	768
	l(−)-Malate	0·02	O_2	1055	623

Quantitative aspects of the effects of fumarate, oxaloacetate and α-ketoglutarate. In the previous experiments acetoacetate and the second substrate were added in approximately molecular proportions. In Tables 7 and 8 experiments are recorded in which the amount of acetoacetate was considerably in excess of the amounts of fumarate or α-ketoglutarate. It will be seen that α-ketoglutarate (Table 7) causes the removal of an equivalent quantity of acetoacetate, while in the case of fumarate and oxaloacetate (Table 8) no simple molecular proportion emerges. One molecule of oxaloacetate, for example, causes the disappearance of up to 4 molecules of acetoacetate. The effects of fumarate and oxaloacetate thus are catalytic.

Table 7. *Effect of α-ketoglutarate on the anaerobic removal of acetoacetate in sheep heart*

(Initial concentration of acetoacetate 0·02 M; N_2; 60 min. incubation. The data refer to 4 ml. at 40°.)

α-Ketoglutarate added (μl.)	0	112	224	448	896
Acetoacetate removed (μl.)	300	420	515	750	870
Extra acetoacetate removed on addition of α-ketoglutarate (μl.)	—	120	215	450	570

Table 8. *Effect of fumarate and oxaloacetate on the anaerobic removal of acetoacetate in sheep heart*

(Initial concentration of acetoacetate 0·036 M; N_2; incubation 120 min.; the data refer to 4 ml. suspension.)

		Fumarate		Oxaloacetate	
Further substrate added (μl.)	None	179	89·5	179	89·5
Acetoacetate removed (μl.)	220	585	470	860	565
Extra acetoacetate removed owing to addition of fumarate or oxaloacetate (μl.)	—	365	250	640	345

Mechanism of action of α-ketoglutarate. The results of two experiments in which approximately equimolecular quantities of acetoacetate and α-ketoglutarate were added anaerobically to minced sheep heart and the changes in the concentrations of relevant metabolites were measured are shown in Table 9. When α-ketoglutarate alone was added, part of it disappeared and equivalent amounts of α-hydroxyglutarate and succinate were formed, in

Table 9. *Acetoacetate and α-ketoglutarate in sheep heart*

Vessel no. ...	(1)	(2)	(3)
Exp. 1. 48 ml. suspension incubated for 30 min. anaerobically			
Metabolites added (μl.):			
Acetoacetate	10,900	10,900	Nil
α-Ketoglutarate	Nil	10,750	10,750
Metabolites found (μl.):			
Acetoacetate	8,620	6,100	Nil
β-Hydroxybutyrate	1,450	3,915	Nil
α-Hydroxy- plus α-keto-glutarate	335	6,810	10,200
Succinate	110	3,990	285
CO_2 plus HCO_3 (formed)	1,800	5,550	1,800
Changes in metabolites (μl.):			
Acetoacetate	−2,280	−4,800	Nil
β-Hydroxybutyrate	+1,450	+3,915	Nil
α-Hydroxy- plus α-keto-glutarate	+ 335	−3,940	− 550
Succinate	+ 110	+3,990	+ 285
CO_2 plus HCO_3	+1,800	+5,500	+1,800
Exp. 2. 48 ml. suspension incubated for 60 min. anaerobically			
Metabolites added (μl.):			
Acetoacetate	Nil	21,990	Nil
α-Ketoglutarate	21,500	21,500	Nil
Metabolites found (μl.):			
Acetoacetate	Nil	13,230	Nil
Succinate	2,005	5,540	415
α-Ketoglutarate	17,350	12,600	Nil
α-Hydroxyglutarate	2,220	2,990	1,005
Changes in metabolites (μl.):			
Acetoacetate	Nil	−8,760	Nil
Succinate	+2,005	+5,540	+ 415
α-Ketoglutarate	−4,150	−8,900	Nil
α-Hydroxyglutarate	+2,220	+2,990	+1,005

accordance with the dismutation described by Weil-Malherbe (1937),

$$2\ \alpha\text{-ketoglutarate} + H_2O = \alpha\text{-hydroxyglutarate} + \text{succinate} + CO_2. \quad (3)$$

When both acetoacetate and α-ketoglutarate were present, a greater amount of α-ketoglutarate disappeared, the excess being approximately equivalent to the extra removal of acetoacetate. At the same time the yield of succinate increased. Acetoacetate was largely recovered as β-hydroxybutyrate. Such acetoacetate as was not recovered could be accounted for by the non-enzymic ketone decomposition. The interaction between acetoacetate and α-ketoglutarate may thus be attributed to the reaction already found by Krebs & Johnson (1937) to occur in kidney and brain:

$$\alpha\text{-ketoglutarate} + \text{acetoacetate} + H_2O = \text{succinate} + CO_2 + \beta\text{-hydroxybutyrate}. \quad (4)$$

Acetoacetate and fumarate. The results of an experiment in which acetoacetate and fumarate were added anaerobically to sheep heart are recorded in Table 10. It will be seen that in vessel 3 the centration during the incubation was therefore much lower than in vessel 1 and the deficit, if any, is therefore expected to be greater in 2 than in 3.

Of the added fumarate 72 % was recovered either as fumarate or as malate in the control vessel 1 (where no other substrate was added). The rest was converted into succinate, α-keto- and α-hydroxyglutarate. This may be explained by a series of oxido-reductions, the primary reaction being:

$$\text{fumarate} + \text{malate} = \text{succinate} + \text{oxaloacetate}.$$

This reaction was observed by Green (1936) and Dewan & Green (1937) in certain enzyme preparations. It would be followed by the series of reactions of oxaloacetate reported by Krebs, Eggleston, Kleinzeller & Smyth (1940) in which part of the oxaloacetate is reduced to malate whilst part enters oxidative reactions yielding the tricarboxylic acids and α-ketoglutarate. The latter then undergoes dismutation (3).

The effect of fumarate on the removal of acetoacetate (vessel 3) is, as in previous experiments,

Table 10. *Acetoacetate and fumarate in sheep heart*

(60 ml. suspension incubated for 90 min. anaerobically (substrate concentration about 0·02 M).)

Vessel no. ...	(1)	(2)	(3)	(4)
Metabolites added (μl.):				
Acetoacetate	Nil	27,420	27,420	Nil
Fumarate	26,880	Nil	26,880	Nil
Metabolites found (μl.):				
Acetoacetate	—	19,800	1,275	Nil
β-Hydroxybutyrate	—	4,410	28,225	Nil
Succinate	3,740	360	5,810	330
α-Ketoglutarate	760	48	1,080	260
α-Hydroxyglutarate	2,100	1,445	2,860	1,170
Fumarate	5,660	Nil	5,330	210
l(−)-Malate	13,660	Nil	10,820	505
Citrate	Nil	Nil	750	Nil
Oxaloacetate	Nil	Nil	Nil	Nil
Pyruvate	Nil	Nil	Nil	Nil
Changes in metabolites (μl.):				
Acetoacetate	—	−7,620	−26,145	—
β-Hydroxybutyrate	—	+4,410	+28,225	—
Succinate	+3,740	+ 360	+ 5,810	—
α-Ketoglutarate	+ 760	+ 48	+ 1,080	—
α-Hydroxyglutarate	+2,100	+1,445	+ 2,860	—
Fumarate + *l*(−)-malate	−7,560	Nil	−10,730	—
Citrate	—	—	+ 750	—

acetoacetate removed agrees within the limits of error with the β-hydroxybutyrate recovered. In the control vessel 2, the yield of β-hydroxybutyrate is somewhat low, but the deficit is of the order expected on account of the ketone decomposition of acetoacetate. The loss by this side reaction is, of course, the greater the lower the rate of acetoacetate metabolism. In vessel 3, acetoacetate was almost completely removed. The average acetoacetate con-

catalytic; the extra amount of acetoacetate removed as a result of the addition of fumarate (difference between vessels 2 and 3) is 18,500 μl. The extra amount of fumarate removed as a result of the presence of acetoacetate (difference between fumarate + malate in vessels 1 and 3) is 3170 μl. Thus 1 molecule of fumarate has caused the disappearance of at least 5·8 molecules of acetoacetate. The effect of fumarate on the removal of acetoacetate can be

satisfactorily explained if it is assumed that the following primary reaction takes place:

malate + acetoacetate
= oxaloacetate + β-hydroxybutyrate (5)

Oxaloacetate formed by this reaction is expected to disappear almost at once (see Krebs, Eggleston, Kleinzeller & Smyth, 1940). It is not possible to formulate exactly the coupled oxido-reductions taking place after addition of fumarate and acetoacetate, as several combinations of the following reactions are feasible:

Reductions:

acetoacetate → β-hydroxybutyrate
α-ketoglutarate → α-hydroxyglutarate
fumarate → succinate

Oxidations:

malate → oxaloacetate
triose or triosephosphate → pyruvic acid
oxaloacetate + pyruvate → tricarboxylic acids (citric, *iso*citric and *cis*-aconitic acids)
tricarboxylic acids → α-ketoglutarate
α-ketoglutarate → succinate

Succinate, it will be noted, can be formed in two different ways. How much is formed by reduction of fumarate and how much by oxidation of α-ketoglutarate can be approximately estimated from the oxidation reduction balance of the system. The calculation is not completely accurate because certain small fractions of some metabolites were not formed during the incubation but were present from the start, as the figures referring to the control vessel 4 indicate. The preformed quantities were not determined in this experiment; being small they were neglected in the calculation of Table 11. The

Table 11. *Oxido-reduction balance of the reactions taking place in sheep heart after addition of fumarate and acetoacetate*

(Based on data for vessel 3 of Table 10. The figures refer to O- (or 2 H-) equivalents, the reductive formation of β-hydroxybutyrate, of β-hydroxyglutarate and of succinate being taken to require 1 O-equivalent, the oxidative formation of citrate being taken to require 3 O-equivalents, of α-keto- and α-hydroxyglutarate 4 O-equivalents, of succinate 5 O-equivalents.)

	O-equivalents (μl.)	Total
Reductions:		
Formation of β-hydroxybutyrate	28,225	
Formation of α-hydroxyglutarate	2,860	33,990
Formation of succinate (half of total)	2,905	
Oxidations:		
Formation of citrate	2,250	
Formation of α-ketoglutarate	4,320	
Formation of α-hydroxyglutarate	11,440	32,540
Formation of succinate (half of total)	14,530	

table shows that oxidations and reductions balance if it is assumed that half of the succinate is formed by reduction and half by oxidation. Another balance sheet comparing the number of fumarate molecules removed with the number of molecules formed from fumarate gives an indication of how much of the pyruvate required for the formation of the tricarboxylic acids (and their oxidation products α-ketoglutarate and succinate) is derived from cell material (carbohydrate) and how much from the ketone decomposition of oxaloacetate. If pyruvate comes from carbohydrate, one molecule of fumarate is needed for each molecule of tricarboxylic acid, or α-ketoglutarate, or succinate. If pyruvate is derived from oxaloacetate, and thus ultimately from the added fumarate, two molecules of fumarate are needed for each molecule of tricarboxylic acid, α-ketoglutarate and (oxidatively formed) succinate. The data given in Table 12 indicate that, in all,

Table 12. *Fumarate used and products of fumarate found in the presence of acetoacetate*

(Based on data for vessel 3 of Table 10.)

	μl.	Total
Equivalents of fumarate removed (not counting those converted into malate)	10,730	—
Equivalents recovered:*		
1. Assuming that 1 mol. fumarate gives 1 mol. end-product:		
Citrate	750	
α-Keto- and α-hydroxyglutarate	2,510	8,740
Succinate	5,480	
2. Assuming that citrate, α-keto- and α-hydroxyglutarate and one half of the succinate require 2 mol. of fumarate, and the other half of succinate 1 mol.:		
Citrate	1,500	
α-Keto- and α-hydroxyglutarate	5,020	14,740
Succinate	8,220	

* Corrected for quantities preformed in the tissue according to vessel 4 (Table 10).

6000 μl. of pyruvate were required for the formation of citrate, α-keto- and α-hydroxyglutarate and succinate. About one-third of this (10,730 − 8740 = 1990 μl.) could have been derived from fumarate. The rest must have come from other sources, probably carbohydrate. Determinations of lactate in the suspensions showed no appreciable difference between controls and those containing fumarate and acetoacetate.

The oxidative formation of citrate, α-keto- and α-hydroxyglutarate and succinate involves a liberation of CO_2. The CO_2 output was measured in a separate experiment recorded in Table 13. These measurements were not very accurate because ketone decomposition of acetoacetate was fairly rapid under the conditions of CO_2 determination. The figures for total CO_2 in Table 13 have been corrected for this decomposition, but are probably

still a little too high. The total CO_2 formation was about 80 % of the β-hydroxybutyrate found, which is of the expected order of magnitude.

Table 13. *Liberation of CO_2 after addition of fumarate and acetoacetate to sheep heart*

(The data refer to 4 ml. suspension incubated anaerobically for 60 min.)

Substrates added (0·02 M final concentration)	Fumarate	Acetoacetate	Fumarate plus acetoacetate
CO_2 plus HCO_3' formed (μl.)	+20	+118	+1017
Acetoacetate removed (μl.)	—	−175	−1153
β-Hydroxybutyrate formed (μl.)	Nil	+219	+1260

Acetoacetate and oxaloacetate. Fumarate in the foregoing experiments can be replaced by *l*(−)-malate without significant effect on the results.

Many experiments were carried out where oxaloacetate replaced fumarate. An example is given in Table 14. The results are in agreement with the view that oxaloacetate does not react as such with acetoacetate, but only after reduction to malate. Oxaloacetate alone forms malate, fumarate and some succinate by reduction, and citrate, α-ketoglutarate, α-hydroxyglutarate and some succinate by oxidation. When acetoacetate is present together with oxaloacetate, the former is reduced to β-hydroxybutyrate and the quantities of malate and fumarate formed are smaller, whilst those of the oxidation products (citrate, α-keto- and α-hydroxyglutarate, and succinate) are increased.

The following two points deserve special mention. (1) The acetoacetate removed is completely accounted for by the β-hydroxybutyrate formed. None of the acetoacetate can therefore have reacted in the manner assumed by Breusch (1943) and Wieland & Rosenthal (1943). (2) In the presence of acetoacetate some of the added oxaloacetate is recovered unchanged. It is unlikely, in view of the instability of this substance, that the recovered oxaloacetate is a remnant of the originally added compound, since no oxaloacetate is found in the absence of acetoacetate. It is more probable that oxaloacetate is found because it is continuously reformed from malate according to reaction (5).

Table 14. *Acetoacetate and oxaloacetate in sheep heart*

(60 ml. suspension, incubated for 80 min. anaerobically; except vessel 4 which was acidified at the start to give the initial quantities of metabolites in the tissue.)

Vessel no. ...	(1)	(2)	(3)	(4)
Metabolites added (μl.):				
Acetoacetate	—	49,800	49,800	—
Oxaloacetate	26,900	26,900	—	—
Metabolites found (μl.):				
Acetoacetate	Nil	19,100	45,600	Nil
Oxaloacetate	Nil	1,275	Nil	Nil
β-Hydroxybutyrate	~Nil	31,500	5,950	~Nil
Succinate	4,690	6,490	240	~Nil
l(−)-Malate	8,660	3,480	~Nil	~400
Fumarate	3,550	3,260	~540	~550
Citrate, *iso*citrate and *cis*-aconitate	850	1,320	340	360
Pyruvate	5,480	2,265	Nil	Nil
Lactate	11,220	9,800	11,480	7,300
α-Ketoglutarate	890	2,430	125	90
α-Hydroxyglutarate	1,490	3,410	590	870
Changes in metabolites (μl.):*				
Acetoacetate	—	−30,700	−4,200	—
β-Hydroxybutyrate	—	+31,500	+5,950	—
Oxaloacetate	−26,900	−25,625	—	—
Succinate	+ 4,690	+ 6,490	+ 240	—
l(−)-Malate	+ 8,260	+ 3,080	− 400	—
Fumarate	+ 3,000	+ 2,710	Nil	—
Citrate, *iso*citrate and *cis*-aconitate	+ 490	+ 960	~Nil	—
Pyruvate	+ 5,480	+ 2,265	—	—
Lactate	+ 3,920	+ 2,500	+4,180	—
α-Ketoglutarate	+ 800	+ 2,340	~Nil	—
α-Hydroxyglutarate	+ 620	+ 2,540	~Nil	—

* Corrected for preformed metabolites as found in vessel 4.

AEROBIC REACTIONS OF ACETOACETATE

The experiments reported so far deal chiefly with the mechanism of the anaerobic reactions of acetoacetate in sheep heart. In the presence of O_2 further reactions occur which will be dealt with in a subsequent paper.

ACETOACETATE IN SHEEP KIDNEY

A number of the foregoing experiments were repeated with minced sheep kidney cortex. The rates of removal of acetoacetate in this tissue were about 50 % of the rates observed in cardiac muscle. The effects of malonate, fumarate, oxaloacetate and α-ketoglutarate were of the same type and of the same order of magnitude. The products of the interaction between these substances and acetoacetate were also the same.

DISCUSSION

The hypotheses of Breusch and of Wieland & Rosenthal. The observations on the anaerobic removal of acetoacetate are accounted for by the reaction (4) and (5) and do not make it necessary to postulate additional anaerobic reactions. No doubt further reactions occur aerobically, as animal tissues can oxidize ketone bodies to completion. The hypotheses of Breusch (1943) and of Wieland & Rosenthal (1943) assume the first step of the oxidative breakdown of acetoacetate to be the anaerobic reactions (1) or (2) respectively. As these reactions do not require O_2 they would be expected to occur under the conditions of our anaerobic experiments if the hypotheses are correct. In fact the reactions did not occur. As virtually all acetoacetate could be recovered either as β-hydroxybutyrate (or unchanged), the occurrence of the reactions (1) or (2) under the conditions of our experiments can be definitely excluded. Breusch and Wieland & Rosenthal did not examine the formation of β-hydroxybutyrate. Their hypotheses were merely based on the observation that acetoacetate increases the yield of citrate formed in the presence of oxaloacetate. However, this observation can be explained otherwise: when oxaloacetate alone is added to the tissue a large proportion undergoes reduction to malic acid, whilst another portion enters oxidative reactions yielding, among other substances, citrate. Acetoacetate causes the reoxidation of malate to oxaloacetate, and thus makes a greater proportion available for the reactions leading to citrate.

Oxidation-reduction potentials. According to Green, Dewan & Leloir (1937), E_0' of the system β-hydroxybutyrate ⇌ acetoacetate is −0·282 V. (38°; pH 7·0). According to Laki (1937), E_0' of the system *l*(−)-malate ⇌ oxaloacetate is −0·169 V. (37°; pH 7·0). Therefore an equilibrium should exist when

$$\frac{[\text{malate}] \times [\text{acetoacetate}]}{[\text{oxaloacetate}] \times [\beta\text{-hydroxybutyrate}]} = 6 \times 10^8.$$

The reaction malate + acetoacetate → oxaloacetate + β-hydroxybutyrate is thus expected to be stopped at a very early stage by the products of the reaction. Actually this does not happen. The fact that oxaloacetate is very rapidly removed by secondary reactions does not account for this apparent discrepancy. Its cause requires further investigation.

SUMMARY

1. Acetoacetate was added to suspensions of minced sheep heart muscle under varying conditions. The chemical changes associated with the disappearance of acetoacetate were investigated.

2. Flasks for large-scale metabolic experiments, holding between 40 and 150 ml. tissue suspension, are described.

3. Manometric methods for the separate determination of oxaloacetate and acetoacetate are described. The former is measured by decarboxylation with aluminium sulphate and the sum of β-ketonic acids by decarboxylation with aniline citrate. Modifications and special details of the methods for the determination of succinic, α-ketoglutaric, α-hydroxyglutaric, fumaric, *l*(−)-malic and β-hydroxybutyric acids are described.

4. $Q_{\text{acetoacetate}}$ was about −15 in O_2 and −6 in N_2 when the concentration of the substrate was 0·02 M and when no other substrates were added. These rates are considerably higher than those previously reported for animal tissues.

5. The aerobic removal of acetoacetate is inhibited by 0·001 M-malonate, whilst the anaerobic removal is not affected. The inhibition is taken to indicate that succinic dehydrogenase plays a part in the aerobic removal of acetoacetate.

6. Fumarate, *l*(−)-malate, oxaloacetate and α-ketoglutarate increase the rates of the aerobic and anaerobic removal of acetoacetate, the latter more than the former. In the presence of these substances the difference between the aerobic and anaerobic rates are small. The same substances largely abolish the inhibition by malonate.

7. The effect of α-ketoglutarate was found to be due to the reaction: acetoacetate + α-ketoglutarate + H_2O = β-hydroxybutyrate + succinate + CO_2.

8. The anaerobic effects of fumarate, *l*(−)-malate and oxaloacetate can be accounted for by the assumption that the reaction: acetoacetate + *l*(−)-malate = β-hydroxybutyrate + oxaloacetate takes place. Oxaloacetate does not appear to react directly with acetoacetate, but only after reduction to *l*(−)-malate.

9. Under the conditions of the anaerobic experiments virtually all metabolized acetoacetate was recovered as β-hydroxybutyrate. This is evidence against the hypotheses of Breusch (1943) and of Wieland & Rosenthal (1943), which assume direct anaerobic formation of citrate from oxaloacetate and acetoacetate. An alternative explanation for the yields of citrate observed by Breusch and by Wieland & Rosenthal is proposed. It is pointed out that the evidence presented by these authors in support of their hypotheses is inconclusive.

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REFERENCES

Auerbach, F. & Krüger, D. (1923). *Z. Untersuch. Nahr.- u. Genussm.* **46**, 97.
Breusch, F. L. (1943). *Science*, **97**, 490.
Dewan, J. G. & Green, D. E. (1937). *Biochem. J.* **31**, 1074.
Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
Edson, N. L. & Leloir, L. F. (1936). *Biochem. J.* **30**, 2319.
Friedemann, T. E. & Graeser, J. B. (1933). *J. biol. Chem.* **100**, 291.
Friedmann, E. & Maase, C. (1910). *Biochem. Z.* **27**, 474.
Green, D. E. (1936). *Biochem. J.* **30**, 629.
Green, D. E., Dewan, J. G. & Leloir, L. F. (1937). *Biochem. J.* **31**, 934.
Hoff-Jorgensen, E. (1940). *Hoppe-Seyl. Z.* **266**, 56.
Jacobsohn, K. P. (1934). *Biochem. Z.* **274**, 167.
Jacobsohn, K. P., Pereira, F. B. & Tapadinhas, J. (1932). *Biochem. Z.* **254**, 112.
Jacobsohn, K. P. & Tapadinhas, J. (1934). *Biochem. Z.* **269**, 225.
Jowett, M. & Quastel, J. H. (1935). *Biochem. J.* **29**, 2181.
Kleinzeller, A. (1943). *Biochem. J.* **37**, 678.
Krebs, H. A. (1932). *Hoppe-Seyl. Z.* **217**, 191.
Krebs, H. A. (1937). *Biochem. J.* **31**, 2095.
Krebs, H. A. (1938). *Biochem. J.* **32**, 108.
Krebs, H. A. (1942). *Biochem. J.* **36**, 303.
Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
Krebs, H. A. & Eggleston, L. V. (1943). *Biochem. J.* **37**, 334.
Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
Krebs, H. A., Eggleston, L. V., Kleinzeller, A. & Smyth, D. H. (1940). *Biochem. J.* **34**, 1234.
Krebs, H. A. & Johnson, W. A. (1937). *Biochem. J.* **31**, 645.
Krebs, H. A., Smyth, D. H. & Evans, E. A. (1940). *Biochem. J.* **34**, 1041.
Laki, K. (1937). *Hoppe-Seyl. Z.* **249**, 63.
Ljunggren, G. (1925). *Katalystik Kolsyreavspjalkning ur ketokarbonsyror*. Lund: A. B. Gleerupska, Univ. Bokhandeln.
Ostern, P. (1933). *Hoppe-Seyl. Z.* **218**, 160.
Pollak, L. (1907). *Beitr. chem. Physiol. Path.* (Hofmeister), **10**, 235.
Quastel, J. H. & Wheatley, A. H. M. (1935). *Biochem. J.* **29**, 2773.
Snapper, I. & Grünbaum, A. (1927). *Biochem. Z.* **181**, 410, 418.
Wakeman, A. J. & Dakin, H. D. (1910). *J. biol. Chem.* **6**, 373; **8**, 108.
Warburg, O. & Yabusoe, M. (1924). *Biochem. Z.* **146**, 380.
Weil-Malherbe, H. (1937). *Biochem. J.* **31**, 2208.
Weil-Malherbe, H. (1938). *Biochem. J.* **32**, 1033.
Wieland, H. & Rosenthal, C. (1943). *Liebigs Ann.* **554**, 241.
Wohl, A. & Claussner, P. (1907). *Ber. dtsch. chem. Ges.* **40**, 2308.
Wohl, A. & Oesterlin, C. (1901). *Ber. dtsch. chem. Ges.* **34**, 1139.

The Effect of Various Chemical and Physical Agents on the Dehydrogenating Enzymes of *Eberthella typhosa*

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Knowledge of dehydrogenating enzymes has become a useful tool in the study of the nutrition of bacteria. The effect of physical and chemical agents on such dehydrogenation systems has been studied by many workers. Quastel & Wooldridge (1927*a*) found that exposure of *Escherichia coli* to various chemical and physical injuries resulted in considerable and partly selective inhibition of the dehydrogenating mechanisms. Cell destruction produced by repeated freezing and thawing has been studied by Young (1929), Yudkin (1937*b*) and Adler, Hellström, Günther & v. Euler (1938). The selective action of many types of injury forms the basis of the theory of dehydrogenating mechanisms formulated by Quastel and co-workers (Quastel, 1926; Quastel & Wooldridge, 1927*b*), which stresses the importance of the structural integrity of the cell.

The present investigation deals with the dehydrogenating mechanisms of *Eberthella typhosa* and the effect of chemical and physical conditions on these enzymes. Previous experiments on the influence of physical and chemical factors on bacterial dehydrogenases have been made on *Esch. coli*; it seemed, therefore, of interest to study these problems in connexion with a pathogenic micro-organism with different fermentative properties.

GENERAL METHODS

The following strains of *E. typhosa* were used: the motile H901 and Vi2, and the non-motile O901. The bacteria were grown on ordinary nutrient agar at 37°. After 24 hr. incubation the cultures were suspended in saline and washed three times. Longer incubation than 24 hr. decreased the dehydrogenating abilities of the bacteria. For the detection of hydrogen donators the procedure previously described (Guggenheim, 1944) was used, methylene blue serving as H acceptor. In spite of carefully controlled conditions the dehydrogenase content found for individual bacterial suspensions varied. Consequently, each substrate was tested four to five times with each of the strains. The results represent average values.